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Citation for published version:

Suku, M, Forrester, LM, Biggs, M & Monaghan, M 2021, 'Resident macrophages and their potential in cardiac tissue engineering', *Tissue engineering*. <https://doi.org/10.1089/ten.TEB.2021.0036>

Digital Object Identifier (DOI):

[10.1089/ten.TEB.2021.0036](https://doi.org/10.1089/ten.TEB.2021.0036)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Tissue engineering

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Tissue Engineering

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Journal:	<i>Tissue Engineering</i>
Manuscript ID	TEB-2021-0036.R1
Manuscript Type:	Review Article - Part B
Date Submitted by the Author:	13-May-2021
Complete List of Authors:	Suku, Meenakshi; University of Dublin Trinity College Forrester, Lesley; University of Edinburgh MRC Centre for Inflammation Research Biggs, Manus; National University of Ireland Galway Monaghan, Michael; University of Dublin Trinity College,
Keyword:	Cardiac Tissue < Applications in Tissue Engineering (DO NOT select this phrase; it is a header ONLY), IPS Cells < Fundamentals of Tissue Engineering (DO NOT select this phrase; it is a header ONLY), Inflammatory/Immune Response < Fundamentals of Tissue Engineering (DO NOT select this phrase; it is a header ONLY)
Manuscript Keywords (Search Terms):	Resident macrophages, Cardiac macrophages, Myocardial Infarction, iPS Cells, Engineered heart tissue
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Resident macrophages and their potential in cardiac tissue engineering

Meenakshi Suku^{1,2,3}, Lesley Forrester⁴, Manus Biggs³, Michael G. Monaghan^{*1,2,3,5}

1. Department of Mechanical, Manufacturing and Biomedical Engineering, Trinity College Dublin, D02 PN40, Ireland
2. Trinity Centre for Biomedical Engineering, Trinity Biomedical Science Institute, Trinity College Dublin, D02 PN40, Ireland
3. CURAM SFI Research Centre for Medical Devices, National University of Ireland, Galway, H91 CF50, Ireland
4. MRC Centre for Regenerative Medicine, University of Edinburgh, 5 Little France Drive, Edinburgh EH16 4UU, UK.
5. Advanced Materials for Bioengineering Research (AMBER) Centre, Trinity College Dublin and Royal College of Surgeons in Ireland, D02 PN40, Ireland

*Corresponding author

Monaghan, Michael G - monaghmi@tcd.ie, +353 1 896 8582

Biggs, Manus - manus.biggs@nuigalway.ie, +353 9 149 4328

Forrester, Lesley - L.Forrester@ed.ac.uk, 0131 651 9553

Suku, Meenakshi - sukum@tcd.ie, +353 877201117

Keywords: Resident macrophages, Cardiac macrophages, Myocardial infarction, iPSCs, Engineered heart tissue

Abstract

Many facets of tissue engineered models aim to understand cellular mechanisms to recapitulate *in vivo* behavior, study and mimic diseases for drug interventions and to provide better understanding towards improving regenerative medicine. Recent and rapid advances in stem cell biology, material science and engineering, have made the generation of complex engineered tissues much more attainable. One such tissue, human myocardium, is extremely intricate, with a number of different cell types. Recent studies have unraveled cardiac resident macrophages as a critical mediator for normal cardiac function. Macrophages within the heart exert phagocytosis and efferocytosis, facilitate electrical conduction, promote regeneration and remove cardiac exophers to maintain homeostasis. These findings underpin the rationale of introducing macrophages to engineered heart tissue, to more aptly capitulate in *vivo* physiology. Despite the lack of studies using cardiac macrophages *in vitro*, there is enough evidence to **accept** that they will be **key** to making engineered heart tissues more physiologically relevant. In this review, we explore the rationale and feasibility of using macrophages as an additional cell source in engineered cardiac tissues.

Impact statement

Macrophages play a critical role in cardiac homeostasis and in disease. Over the last decade, we have come to understand the many vital roles played by cardiac resident macrophages in the heart, including immunosurveillance, regeneration, electrical conduction and elimination of exophers. There is a need to improve our understanding of the resident macrophage population in the heart *in vitro*, to better recapitulate the myocardium through tissue engineered models. However, obtaining them *in vitro* remains a challenge. Here, we discuss the importance of cardiac resident macrophages and potential ways to obtain cardiac resident macrophages *in vitro*. **Finally, we critically discuss their potential in realizing impactful in vitro models of cardiac tissue and their impact in the field.**

Introduction

Macrophages are present in almost every vertebrate tissue. They contribute to the maintenance of immunity by phagocytosing microbes, clearing senescent cells and promoting homeostasis by repairing and regulating the functions of organs. Initially perceived as a cell population only present in, and originating from the blood, recent studies have brought to light the stark heterogeneity in blood-derived and a pre-natal embryologically derived macrophage populations. Most tissues in the body contain a resident macrophage population exhibiting diverse phenotypes, specific to that tissue and niche¹. This phenotypic heterogeneity is potentially due to the impact of the resident tissue environment as well as to differences in the macrophage origin². However, despite being best known as the key mediators in tissue defense, the exact role of different subsets of resident macrophages in protecting the body from pathogens or environmental change are still unclear, as they are hard to extract, purify and study in vitro.

The focus of this review, cardiac resident macrophages; originate during embryogenesis and populate the heart prenatally³. Only within the past 3-4 years, seminal studies unravelling the functions and the physiological importance of cardiac macrophages have emerged (in mice), identifying these cells as a potential candidate in tissue engineering applications. In addition to performing critical functions in healthy myocardium, cardiac resident macrophages respond uniquely to subtle changes in the homeostatic equilibrium. Although there has been a limited number of studies focusing on the role of resident macrophage populations in the heart, there exists enough groundwork to explore the possibilities of achieving more humanized cardiac tissue models using resident macrophages. Additionally, by incorporating resident macrophages within engineered heart tissues, models can be more physiologically relevant, with applications in regenerative medicine, disease modelling and drug screening. However,

there remain a lot of questions to be answered - what degree of these cells are lost during myocardial disease/ischemia; how do they function in response to injury and disease, and what role do their paracrine factors exert in tissue engineered models, to name but a few. We discuss cardiac macrophages in healthy myocardium, and during myocardial infarction (MI) and post-MI heart failure. We recap on the various engineered heart tissue (EHT) models that have emerged over the years and discuss the potential applications of macrophages within such models in cardiac tissue engineering. Finally, we discuss the challenges of obtaining cardiac resident macrophages *in vitro*, and avenues of obtaining them *in vitro* for tissue engineering - such as using induced pluripotent stem cell (iPSC) derived macrophages (iMacs), opening up the possibility to study genes and genetic pathways involved in their function.

Macrophage Populations in the heart and their functions

The cellular content of human myocardium (in descending order by number) predominantly consists of cardiomyocytes, fibroblasts, pericytes and smooth muscle cells, endothelial cells and immune cells⁴. In the murine heart, macrophage populations can be discerned based on their expression of C-C chemokine receptor type 2 (CCR2), major histocompatibility complex-II (MHC-II), T-cell immunoglobulin and mucin domain containing 4 (TIMD4) and Lymphatic Vessel Endothelial Hyaluronan Receptor 1 (LYVE1). Four populations of macrophages have been identified in the murine heart⁵ (Fig.1). The minority are two subsets of CCR2⁺MHC-II^{high} macrophage populations derived from hematopoietic stem cells (HSCs), whereas the most dominant subset, cardiac resident macrophages derived from the yolk sac, are CCR2⁻MHC-II^{low}TIMD4⁺LYVE1⁻. There also exists a fourth subset that consists of CCR2⁻MHC-II^{high}TIMD4⁺LYVE1⁺ macrophages which are likely derived from CCR2⁺MHC-II^{low} macrophages during postnatal development^{5, 6} and partially maintained by CCR2⁺MHC-II^{high} macrophages. CCR2⁺MHC-II^{high} macrophages populate the heart soon after birth and are maintained through monocyte recruitment and proliferation⁷. Also, under homeostasis, cardiac

macrophages continually self-renew with a turnover period of almost five weeks⁸. Recently this same CCR2⁺ and CCR2⁻ macrophage distinction has been identified in humans⁹, with the yolk sac-derived CCR2⁻ human counterpart showing CD14⁺CD45⁺CD64⁺CCR2⁻HLA-DR^{high} expression^{5, 9}. In a more recent study, Tucker *et al.* reported two cardiac macrophage subsets within the human heart which were positive for CD163, COLEC12, mannose receptor MRC1, E3 ubiquitin ligase MARCH1 and natural resistance-associated macrophage protein 1 (NRAMP1). While both of the identified subsets express anti-inflammatory (M2) polarization-associated genes, one subset particularly expressed RBPJ, F13A1 and the other expressed transmembrane collagen COL23A1¹⁰.

CCR2⁺ and CCR2⁻ macrophage populations are present in specific locations within the heart, performing distinct functions. CCR2⁺ macrophages are usually found in the trabecular projections of the heart and their role in cardiac development **has not been fully delineated**. However, they are activated when homeostatic balance is interrupted- such as during a disease¹¹. In contrast, CCR2⁻ macrophages, herein referred to as cardiac resident macrophages, occupy the myocardial wall and play a principal role in normal coronary development and maturation¹². However, such studies are mostly limited to murine models. In addition to demonstrating robust phagocytic behavior¹³, cardiac resident macrophages have demonstrated a role in neonatal cardiac regeneration in mice, salamanders and zebrafish¹⁴⁻¹⁶, potentially through the stimulation of angiogenesis¹⁷.

Furthermore, cardiac resident macrophages interact very closely with cardiomyocytes and form signaling complexes through connexin-43 allowing synchronous depolarization¹⁸ (**Fig.2**). Hulsmans *et al.* have shown that depletion of either cardiac resident macrophages or connexin-43 effects a delay in conduction and blockage in the atrioventricular node in mice. This study highlights a critical role of cardiac macrophages in coupling with cardiomyocytes to facilitate electrical conduction within the heart. Undoubtedly, the coupling of cardiomyocytes and

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cardiac resident macrophages holds a lot of possibilities in regenerative medicine, to be explored in the future.

In addition to their involvement in the conduction system of the heart, cardiac macrophages also play a role in phagocytosis and efferocytosis¹⁹ (Fig.2). Cardiac macrophages express high levels of myeloid epithelial reproductive tyrosine kinase (Mertk), which plays a role in macrophage-mediated efferocytosis. In the absence of this protein, cell debris removal during cardiac repair is severely affected, resulting in increased infarct size and reduced cardiac function²⁰. Removal of debris and other materials ejected by cardiomyocytes is crucial in maintaining adequate cardiac function even in non-pathological settings. Similar to how the central nervous system disposes of dysfunctional organelles²¹, cardiomyocytes remove dysfunctional subcellular particles with the help of cardiac resident macrophages²². Likewise, Nicolas-Avila *et al.* documented that cardiomyocytes, which are continuously contracting and metabolically active cells, release subcellular particles termed “exophers” containing defective mitochondria²². These exophers are taken up by cardiac resident macrophages through Mertk to maintain cardiac homeostasis. This maintenance role of cardiac resident macrophages is further demonstrated when they are depleted in the myocardium (by intraperitoneal injection of 10 µg/kg diphtheria toxin in mice)²², which in turn reduces the presence of these exophers, delineating the role of cardiac resident macrophages in maintaining normal cardiac autophagy. In addition to unveiling this unique role of cardiac resident macrophages, this is the first study to show that myocardial tissue releases exophers (in mice).

Macrophage Populations during MI and post-MI heart failure

In the murine heart, the macrophages and monocytes population in the myocardium following an infarct is dramatically different from that in steady state (Fig.3). The temporal dynamics of leukocyte infiltration following surgically induced MI has been assessed using flow cytometry and determines that neutrophils are among the first leukocytes to reach ischemic heart tissue²³.

This migration of leukocytes to the ischemic site is mediated by C-X-C motif chemokine ligand 2 (CXCL2) and CXCL5 production by CCR2⁺ macrophages²⁴. Monocyte recruitment to the infarct site begins as early as 30 minutes depending on CCR2 signaling, from both the bone marrow and the spleen, via MCP-1/CCR2 signaling²⁵. This infiltration of monocytes and macrophages to the infarcted tissue occurs in two phases²³. The Ly-6C^{high} monocytes exhibiting pro-inflammatory phenotype predominate the early days (marking the first phase), peaking on day 3²³ (Fig.3). Around 40% of the infiltrating Ly-6C^{high} monocytes in the first 24hrs post-MI are from the splenic monocyte reservoir²⁶. During this period, also known as the inflammatory phase, macrophages and neutrophils clear dead cells and debris. After proper resolution of the inflammatory phase, there occurs a phenotypic transition from Ly-6C^{high} monocytes to Ly-6C^{low} monocytes and M2 macrophages which exert reparative functions and renew locally²⁶. Ly-6C^{low} macrophages predominate the later stages as inflammation resolves (marking the second phase), peaking between days 5 to 7 post-infarct²⁷ (Fig.3). Similarly, the remote myocardium also undergoes a sequential Ly-6C^{high} to Ly-6C^{low} differentiation pattern, with the Ly-6C^{low} cells peaking 5 days later than at the infarct²⁸. The cardiac resident macrophages in the infarct area die shortly after MI⁶. However, more recent studies show that in the post-MI phase, the majority of the cardiac resident macrophages proliferate locally with a minor contribution from blood-derived monocytes⁶.

During the inflammatory phase, classically activated (M1) macrophages secrete cytokines such as tumor necrosis factor α (TNF α), interleukin (IL)-1 β , IL-6 and matrix metalloproteinases (MMPs) to facilitate extracellular matrix (ECM) breakdown²⁹. Phagocytosis mediated by M1 macrophages is crucial for the initiation of the wound healing process. However, prolonged production of these inflammatory cytokines could lead to a number of pathological consequences including cardiomyocyte death, cardiac rupture, fibrosis, dilated cardiomyopathy due to matrix degradation etc.³⁰. For example, TNF α (produced by

macrophages, cardiomyocytes and endothelial cells) induces cardiomyocyte hypertrophy which can lead to post-MI heart failure³¹. Over the following days, macrophages promote fibroblast differentiation into myofibroblasts which express smooth muscle actin and lay down collagen, contributing to scar formation³². Additionally, **vascular endothelial growth factor (VEGF) and transforming growth factor- β (TGF- β)** produced by the macrophages facilitate revascularization of tissues^{23, 33}. Finally, macrophages also produce MMPs that help in breaking down the ECM and promote tissue remodeling.

Any abnormalities in either of the phases can be detrimental to the cardiovascular system. In particular, macrophage depletion is deleterious to infarct healing and worsens the remodeling process^{34, 35}. Despite being merely 2-5% of the total macrophage population in the heart in the initial weeks post-MI, the depletion of cardiac resident macrophages impaired normal infarct healing and adversely affected the remodeling process⁵. Shirishi *et al.* demonstrated the effect of inactivation of **alternatively activated (M2)** macrophages post-MI by using a mouse model with Trib1 gene deletion³⁶. The impaired wound remodeling was rescued by the introduction of M2 macrophages exogenously. Additionally, limiting the number of infiltrating monocytes via CCR2 blockage has shown to decrease the infarct size in MI recovery animal models³⁷⁻³⁹, demonstrating the relevance of therapeutic approaches to limit infiltrating monocyte numbers in patients with increased systemic inflammatory activity to improve post-MI recovery.

As mentioned previously, initial fibrosis following MI is necessary to facilitate scar formation and initiate tissue remodeling. However, extensive fibrosis can result in morbid cardiac remodeling and heart failure⁴⁰. The macrophage number in the heart progressively increases from the onset of MI to heart failure, with local proliferation as well as recruitment responsible⁴¹. While macrophages in the remote myocardium increase, their number depletes at the site of infarct⁶. This local expansion of cardiac macrophages aggravates the progression of heart failure by producing factors that promote fibrosis, such as TGF- β , platelet derived

growth factor and angiotensin-II⁴². Myofibroblasts and cardiomyocytes also further prompt TGF- β production causing adverse remodeling that leads to heart failure. The detrimental effects of recruited monocytes/macrophages are further recognized as splenectomy reduces infiltrating macrophages and improves cardiac function⁴³. A recent report also suggested that mechanical strain can trigger macrophage proliferation by **activating mitogen activated protein kinase (MAPK)** pathway, demonstrating that increased higher mechanical strain in failing heart could stimulate macrophage expansion⁶. These evidences further highlight the ill impacts of infiltrating macrophages in the infarcted heart. **It is also of importance to note recent studies suggesting that the opportunity for successful regeneration of the heart after MI is restricted to 3 days post-MI and is potentially mediated by cardiac resident macrophages and fibroblasts⁴⁴.**

Engineered Heart Tissue Models

Tissue engineering considerably enables our understanding of disease biology and possibilities for therapeutic intervention⁴⁵. As opposed to 2D cell culture models, tissue engineered cardiac models provide the benefit of mimicking *in vivo* myocardium, with the additional functional benefit of tissue contraction, which is inherent to cardiac function⁴⁶. Moreover, it also comes with the ethical benefit of using lesser number of animals. Engineered constructs are typically generated using primary cardiomyocytes (adult, neonatal, fetal), as well as cardiomyocytes obtained through iPSCs or embryonic stem cells (ESCs)⁴⁷. Although engineered heart tissues (EHTs) can be fabricated purely using cardiomyocytes, the contractile function and the complexity of native myocardium is better recapitulated with the addition of non-myocytes such as fibroblasts and endothelial cells⁴⁸. Other non-myocytes that have been explored include cardiac stem cells⁴⁹.

Over two decades ago, the first EHT model was fabricated with chick embryonic cardiomyocytes mixed with Matrigel which anchored on to opposite ends of two Velcro coated glass tubes⁵⁰ (Fig.4). This culture provided mechanical strain to the engineered tissue and hence

was utilized as a platform for understanding the effects of uniaxial and multiaxial stretching, growth factors, cardiomyocyte maturation etc. This same principle was used to modify the geometry of the EHT to obtain a ring-shaped model which supported mammalian (rat) cardiomyocytes and also facilitated long-term force measurements^{51, 52}. Although a number of different scaffold-based cardiac tissue engineered models were developed around this period, the next major modification to the EHT design was miniaturized EHTs made with fibrin, that were adapted to 24-well format, to facilitate drug screening⁵³. This study demonstrated the use of video-optical recording for the measurement of contractility of the tissues, which is a commonly used method of contractility measurement at the moment⁵³. Shortly after, Boudou *et al.* generated collagen/fibrin matrix-based cardiac microtissues using microfabricated tissue gauges that incorporate microelectromechanical systems (MEMS) cantilevers⁵⁴. This facilitated in simultaneously constraining and reporting forces generated by the tissues in real time⁵⁴. Similar approaches with cantilevers to allow tissue anchorage has also been used by Turnbull *et al.* to facilitate fabrication of potential preclinical *in vitro* myocardium models⁵⁵. Another interesting modification to the EHT came with the use of a surgical suture within PDMS molds to obtain aligned cardiac tissues with striations called 'Biowire'⁵⁶. Following this study, Bian *et al.* demonstrated that fabricating cardiac tissues with longer elliptical pores within the patch networks improve ECM alignment within the patch⁵⁷. In the past 5 years, the field of EHTs further underwent drastic advances where fibrin based EHTs under dynamic culture (using platform rockers) showed improvement in tissue maturation⁵⁸. These EHTs, called 'cardiobundles' were designed to be cylindrically shaped and anchored within porous flexible nylon frames that supported chronic auxotonic loading and free-floating culture of the tissues⁵⁸. More recently, the advanced successor of Biowire, named 'Biowire II', a heteropolar cardiac tissues containing distinct atrial and ventricular ends was generated⁵⁹. This heteropolar tissue formation was achieved using directed cell differentiation and electrical field

conditioning⁵⁹. With over two decades of input, one of the most recent advancement in EHTs is a mesh structured EHT patch of dimension 5 x 7cm, that closely resemble human ventricular tissue⁶⁰.

One of the key features of EHTs is their ability of respond to drugs. Most of the different types of EHTs fabricated over the years aim to feature their drug response. For example, over a decade ago, Hansen *et al.* showed that EHTs display a concentration-dependent increase in relaxation time on treatment with chromanol, quinidine, and erythromycin, while doxorubicin treatment resulted in decreased contractile force ⁵³. Similarly, Boudou *et al.* tested isoproterenol and digoxin on their EHTs to understand the functional consequences of pharmacologic agents⁵⁴. They showed that small doses (10 and 100 nM) of isoproterenol increased the contractility of the EHTs while 1 and 10 mM decreased the contractility⁵⁴. On the other hand, digoxin improved the contractility at 1 nM and 1 mM but was found to be cardiotoxic at 10 mM concentration⁵⁴. Hence, the varied and dose dependent sensitivity of EHTs towards different drugs make them an exciting alternative to animal models in addition to being a useful tool in understanding the effect of various drugs on the myocardium. Despite the proven efficacies of these EHTs, there still remain challenges in making them more humanized, including the addition of right cell types to mimic the complexity of the myocardium. For a more detailed review capturing EHT the reader is referred to the paper by Christian Zuppinger ⁶¹. One of the potential additions to improve the response of EHTs would be cardiac resident macrophages, as they are critical in the normal functioning of the heart.

Macrophages in Tissue Engineering

In recent years, the critical importance of incorporating macrophages into engineered constructs is being recognized^{62,63} and aside from the immunological responses of host macrophages, their role has been harnessed in a number of tissue engineering approaches to

improve tissue maturation, repair and regeneration⁶⁴. Understanding macrophage responses during biomaterial design^{65, 66}, as well as exploring the effects of mechanical cues on macrophage polarization are other areas within tissue engineering that have gained attention^{67, 68}. Researchers have studied the potential of immune assisted tissue engineering, by incorporating macrophages into tissue engineered constructs (Table 1). In particular, macrophages have been used to vascularize biomaterials⁶⁹, by promoting angiogenesis⁷⁰ and vessel formation⁷¹. Although immune assisted tissue engineering has not been largely investigated, these data show the **significance of macrophages in various tissue engineered constructs**.

Within cardiac tissue engineering, the specific functional requirement of this tissue includes contraction, which is inherent to cardiac function⁴⁶. As macrophages have been shown to be integral during signal propagation (See Fig.2), their inclusion in engineered tissue could facilitate robust and more physiologically accurate contraction mechanics. Engineered constructs are typically generated using of primary cardiomyocytes (adult, neonatal, fetal), as well as cardiomyocytes obtained through iPSCs or embryonic stem cells (ESCs)⁴⁷. Since the critical role played by macrophages in cardiac regeneration, maintenance of homeostasis and disease progression has been recognized (in mice), cardiac tissue engineering has begun to focus on incorporating macrophages into engineered heart tissues to recapitulate native tissue functions. A pioneering *in vitro* study in which human peripheral blood-derived macrophages and iPSC-derived cardiomyocyte interaction was assessed, attempted to recapitulate critical cellular events during myocardial injury, using a 3D inversion assay. They proposed a bone morphogenetic protein (BMP) mediated crosstalk between these two cell types⁷². Cardiomyocyte-derived BMP promoted pro-inflammatory (M1) macrophage recruitment and M1 macrophage-derived BMP improved cardiomyocyte proliferation and differentiation

potential. This proposed paracrine mechanism of BMPs was new and provided a new insight into the mechanism of interaction between cardiomyocytes and peripheral blood-derived macrophages. Interestingly, a recent study has shown that the presence of macrophages in engineered cardiac tissue facilitates cardiomyocyte de-differentiation and remodeling⁷³. In this study, it was observed that during the initial days (up to 3 days), the macrophages in the engineered cardiac tissue were polarized to an M1 state (CD86 positive), followed by a predominance of M2 macrophages (CD206 positive) as the cardiomyocytes de-differentiated. The effects of macrophage activity on the engineered cardiac tissue model could not be explained in this study however, as a heterogenous population of whole cells from rat heart was used to make the engineered cardiac tissue. Nonetheless, future studies into the influence of macrophage populations of different ontological origin on cardiac function will shed light on their potential role in cardiac tissue engineering.

One study, by Wrona et al. explored the response of human hESC-derived cardiomyocytes to peripheral blood derived monocytes, with a focus on investigating the effects of polarized macrophages and their activation inducing cytokine profiles on cardiomyocyte function⁷⁴. In this study the gene expression profile of the macrophages remained unaffected upon exposure to hESC-derived cardiomyocytes while cardiac-specific genes were downregulated on exposure to M1 and M2 polarized macrophages⁷⁴. Tying in with the findings of this work, a study by Hitscherich et al. using murine ESC-derived cardiomyocytes also showed a decrease in the expression of cardiac-specific genes such as cardiac troponin T and sarcoplasmic/endoplasmic reticulum calcium ATPase when cultured with conditioned media from M1 polarized blood-derived murine macrophages⁷⁵. Here a co-culture system was employed, to demonstrate that M1 macrophages promoted a higher Ca^{2+} fractional release and both macrophage subtypes downregulated the store-operated Ca^{2+} entry in murine ESC-derived cardiomyocytes. As discussed previously, EHTs and other scaffold-based tissue engineered

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models for the myocardium heavily rely on cardiomyocytes, cardiac fibroblasts and endothelial cells to represent the myocardium. Although literature shows the potential of EHTs in guiding us towards obtaining robust *in vitro* models of human myocardium that has the ability to mature, beat in synchrony and respond to drugs, the addition of macrophages into these models could benefit our understanding on the *in vitro* behavior of macrophages in a complex, multicellular humanized setting. From the available *in vivo* studies (in mice), it could be anticipated that the incorporation of cardiac resident macrophages within an EHT (containing cardiomyocytes) will facilitate improved contractility and electrical conduction of the tissue. Additionally, the tissue could be hypothesized to exhibit improved maturity as there could be constant exophers elimination and maintenance of homeostasis.

Another interesting avenue that can be pursued using an EHT model that incorporates cardiac resident macrophages is screening anti-inflammatory drugs for post-MI treatment. Although clinical studies on drug treatment in MI patients is limited, there are a number of drugs that have been developed for regulating immune response post-MI⁷⁶. This includes non-steroidal anti-inflammatory drugs, glucocorticoids, cyclosporine etc⁷⁷. Despite showing an improvement in mortality, these broad anti-inflammatory drugs often fail to provide a cardio-protective effect⁷⁷. To circumvent this limitation, targeted anti-inflammatory drugs can be used. For example, the CANTOS (Canakinumab Anti-Inflammatory Thrombosis Outcomes Study) trial was the first large cardiovascular clinical trial to target the inhibition of inflammation by using the drug canakinumab, which inhibits IL-1 β to subsequently reduce the production of IL-6 and high sensitivity C-reactive protein (hsCRP)⁷⁸. Among the 10,061 patients with previous MI and hsCRP level ≥ 2 mg/l, patients treated with 150 and 300 mg of canakinumab experienced a 15% reduction in major adverse cardiovascular events in comparison with placebo⁷⁸. Although canakinumab was not ultimately approved for cardiovascular treatment, CANTOS trial demonstrated that targeting the inflammatory pathway involving IL-1 β and hsCRP reduces

adverse cardiovascular events. CANTOS was followed by CIRT (Cardiovascular Inflammation Reduction Trial) in which 4786 patients with stable atherosclerosis with diabetes mellitus or metabolic syndrome were subjected to low-dose methotrexate⁷⁹. Low-dose methotrexate failed to reduce major adverse cardiovascular events, and also failed to reduced plasma levels of IL-1 β and hsCRP, further demonstrating the importance of adequate inhibition of IL-1 β to IL-6 pathway for long-term cardiovascular benefits⁸⁰. Further large cardiovascular outcome trials targeting the inflammatory pathways that followed includes COLCOT (Colchicine Cardiovascular Outcomes Trial)⁸¹ and LoDoCO2 (Low Dose Colchicine after Myocardial Infarction)⁸². Both of these trials used low doses of colchicine (0.5mg/day), which is a repurposed anti-inflammatory agent. While COLCOT had 4745 patients with recent MI (less than 30 days), LoDOCO2 had 5522 patients with chronic coronary artery disease and both the studies showed reduction in the incidence of adverse cardiac events such as MI, stroke, cardiovascular death etc^{81, 82}. However, colchicine being associated with increase in pneumonia, nausea and flatulence poses risk⁸⁰. To that end, the lack of success for most of these drugs could also stem from the fact that they are not thoroughly validated *in vitro* prior to human trial. The trajectory of recent and ongoing cardiovascular outcome trials show an increased interest towards novel anti-inflammatory and anti-cytokine drugs⁸⁰. This further demonstrates the benefit of an *in vitro* model of the myocardium that has innate immune cells (especially macrophages) as they serve as the primary target for these drugs and can potentially fill the gap between the patient and the pharmaceutical industry.

iPSC and ESC-derived macrophages

Obtaining human cardiac macrophages for subsequent *in vitro* studies is challenging. To date, macrophage studies have heavily relied on murine or human blood-derived monocytes but these cells are not ideal for appropriating tissue resident phenotypes due to their vast

transcriptomic and epigenetic variations, as well as difference in origin between blood-derived and resident macrophages⁸³. Obtaining primary resident macrophages from vital organs (cardiac macrophages, Kupffer cells, microglia etc.) is not feasible, as acquiring tissue samples from healthy donors is often associated with donor site morbidity, in addition to ethical considerations. To circumvent these limitations, the use of iPSCs and hESCs as macrophage progenitor sources has come to the forefront (Fig.5) (Table 2). Over the last decade, a number of different protocols have been established for stem cell culture and differentiation, to obtain CD14^{high}CD16^{low}CD163⁺CD11b⁺ macrophages, morphologically similar to blood-derived macrophages⁸⁴⁻⁸⁸. Critically, iPSC and hESC-derived macrophage sources have shown therapeutic benefits in ameliorating liver fibrosis⁸⁹, acute lung injury⁹⁰ and are also under clinical trial for liver cirrhosis⁹¹.

Furthermore, recent studies have shown that iMacs share ontogeny with tissue resident macrophages and are independent of MYB^{90, 92, 93}, suggesting that iMacs in isogenic co-cultures may offer a facile and effective approach to obtaining physiologically relevant resident macrophages⁹⁴. For example, iMacs in co-culture with neural cells were found to become microglia-like cells^{95, 96}. Haenseler *et al.* showed that iMacs in co-culture with iPSC-derived neurons, expressed markers relevant to microglia as well as major neurodegenerative disorders⁸⁴. Similarly, iMacs in co-culture with hepatocytes were found to show Kupffer cell-like characteristics⁹³.

In addition to co-culture models, the effect of the local biochemical environment on the specialization of tissue-resident macrophages is becoming more evident in regenerative medicine. Certain transcriptional factors regulate the expression of tissue-resident macrophages⁹⁷, such as Spi-c for the development of the red pulp macrophages in spleen⁹⁸, Sall1 for microglia⁹⁹, and Id3 for Kupffer cells¹⁰⁰. In a more recent study, activation of iMacs

with transcriptional factor KLF1 was found to drive them towards an erythroblastic island like phenotype¹⁰¹. These studies demonstrate that physiologically relevant resident macrophages could be obtained *in vitro* by providing tissue specific cues or by activating specific transcriptional factors. Although, cardiac resident macrophages have not yet been derived from iPSCs, research is proceeding in the right direction, paving way to understanding optimal methods of cardiac resident macrophage acquisition *in vitro*, such as using primary cardiomyocyte conditioned media on iPSC-derived macrophages, establishing co-culture models of cardiomyocytes and iPSC-derived macrophages or using bioinformatics tools to determine transcriptional factors for their induction. Taken together, iPSC-derived macrophages offer great potential in understanding and modelling tissue resident macrophages.

Conclusion

From regarding macrophages as a system of clearing dead cells, to appreciating their role in tissue defense, homeostasis, regeneration and organ development, our understanding of macrophages has advanced significantly in recent years. Lineage tracing and fate mapping studies demonstrated that most vital organs are largely populated by resident macrophages at the onset of embryological development and play critical roles in health and disease. Within the heart, resident macrophages ensure homeostatic cardiac function by performing a number of vital functions including phagocytosis, efferocytosis, eliminating exophers, facilitation of electrical conduction etc. Our understanding of cardiac immunology has progressed well enough to regard cardiac resident macrophages a potential candidate to include in engineered heart tissues, to further our understanding of cell interactions in addition to making the engineered tissues more physiologically relevant. But much more still remains to be

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understood, such as what degree of these cells are lost during myocardial disease/ischemia; how do they function in response to injury and disease, their paracrine effects in tissue engineered models etc. The next step in cardiac tissue engineering is to unravel the crosstalk between cardiac macrophages and cardiomyocytes *in vitro* and further our understanding the molecular processing involved in their interaction and their potential in regenerative medicine, therapeutics, disease modelling and drug screening. With engineered heart tissues gaining great research interest, it is highly pertinent to include a resident macrophage population to make the tissue more physiologically relevant. By building on our strong foundational knowledge on different macrophage populations and with the assistance of new technologies, we will hopefully see rapid progress in finding new therapeutics using cardiac tissue engineering.

Acknowledgement

Funding: This work was supported by EPSRC and SFI Centre for Doctoral Training in Engineered Tissues for Discovery, Industry and Medicine, Grant Number EP/S02347X/1. This publication has emanated from research supported in part by a grant from Science Foundation Ireland (SFI) and the European Regional Development Fund (ERDF) under grant number 13/RC/2073.

Conflict of Interest Statement

The authors declare no conflict of interest.

Authorship Confirmation Statement

All co-authors have reviewed and approved of the manuscript prior to submission. The manuscript has been submitted solely to this journal and is not published, in press, or submitted elsewhere.

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Fig.1 Macrophage subsets in human and mouse and their origin. Cardiac macrophages populate the heart prenatally from the embryonic yolk sac, while bone marrow-derived macrophages enter the heart two weeks after birth. In humans, the macrophage populations are distinguished based on the presence of CCR2, as CCR2-HLA-DR^{high} (cardiac macrophages)

and CCR2⁺HLA-DR^{high} (bone marrow-derived macrophages). The three distinct populations of macrophages in murine heart are, CCR2⁻MHC-II^{low} (cardiac macrophages), CCR2⁻MHC-II^{high} (derived postnatally from cardiac macrophages), CCR2⁺MHC-II^{high} (bone marrow-derived macrophages). *Created with BioRender.com*

Fig.2 Functions of cardiac macrophages in healthy myocardium. In steady state, cardiac macrophages perform several important functions in the myocardium. One of their major functions is to facilitate electrical conduction by coupling with cardiomyocytes through connexin-43 gap junctions. They help in sustaining normal cardiac autophagy by capturing and eliminating exophers (containing defective mitochondria) released by cardiomyocytes, through Mertk. They also perform immunosurveillance and clear dead cells and debris in the myocardium, to maintain homeostasis. The presence of cardiac macrophages is found to be essential in promoting cardiac regeneration. *Created with BioRender.com*

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Tables

Table 1 Examples of macrophage use in tissue engineering

Tissue	Macrophage	Application
Bone	Blood-derived macrophages	Vascularization of scaffold ⁶⁹
	Blood-derived macrophages	Osteogenesis ^{102, 103}
Skeletal muscle	Blood-derived macrophages	Regeneration ⁶³
Blood vessel	Blood-derived macrophages	Vascularization of scaffold ⁷¹
Liver	Kupffer cells	Hepatotoxicity ¹⁰⁴
	Kupffer cells	Disease-drug interaction ¹⁰⁵
Nerve	Microglia	Regeneration ¹⁰⁶
Brain/spinal cord	Microglia	Understand cellular crosstalk during neuroinflammation ¹⁰⁷
Lungs	Alveolar macrophages	Understanding response to atmospheric particles ¹⁰⁸
	Alveolar macrophages	<i>In vitro</i> lung inflammation platform ¹⁰⁹

	Alveolar macrophages	<i>In vitro</i> model for air-blood barrier ¹¹⁰
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Table 2. Examples of iPSC-derived macrophage subsets and their respective phenotypic markers

Tissue	Cell-type	Phenotypic markers
Blood	Blood-derived macrophages	CD18+, CD11b+, CD11c+, CD14+, CD16+, CD115+, CD1a-, CD83-, CD3-, CD19- ¹¹¹ CD14+, CD16+, CD163+, CD86+ ¹¹² CD45+, CD11b+, CD14+, CD163+, CD68+ ¹¹³
Lungs	Alveolar macrophages	CD80+, SIRPa+, MHCII-, Langerin- ⁹⁰
Liver	Kupffer cells	CLEC-4F+, ID1+ and ID3+ ⁹³
Brain	Microglia	IBA1+, CD11c+, TMEM119+, P2RY12+, CD11b+, and CX3CR1+ ¹¹⁴ CD11b+, ITGB2+, CSF1R+, CD45+, IBA1+ and ADORA3+ ¹¹⁵

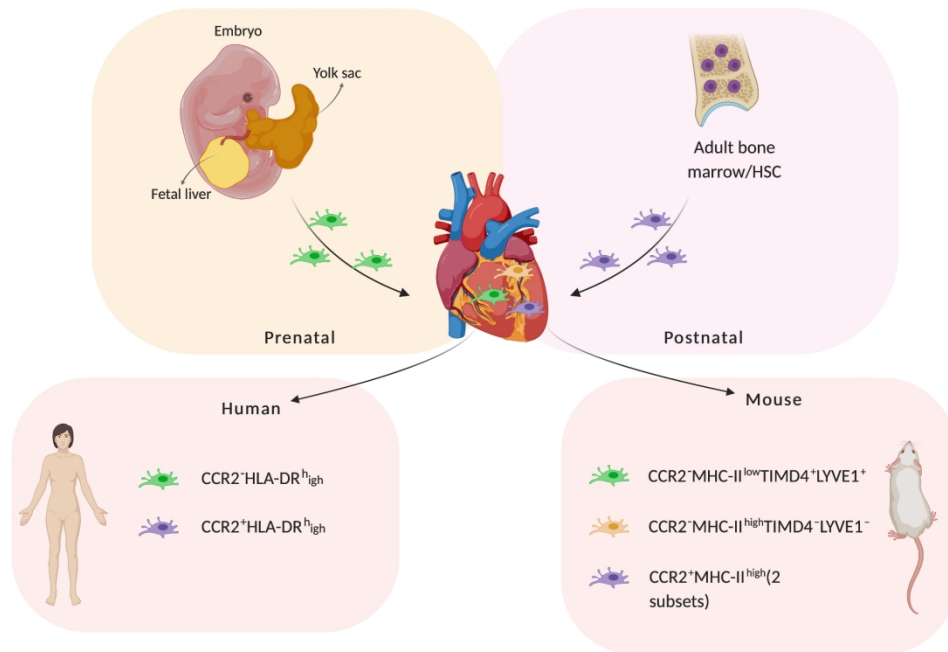


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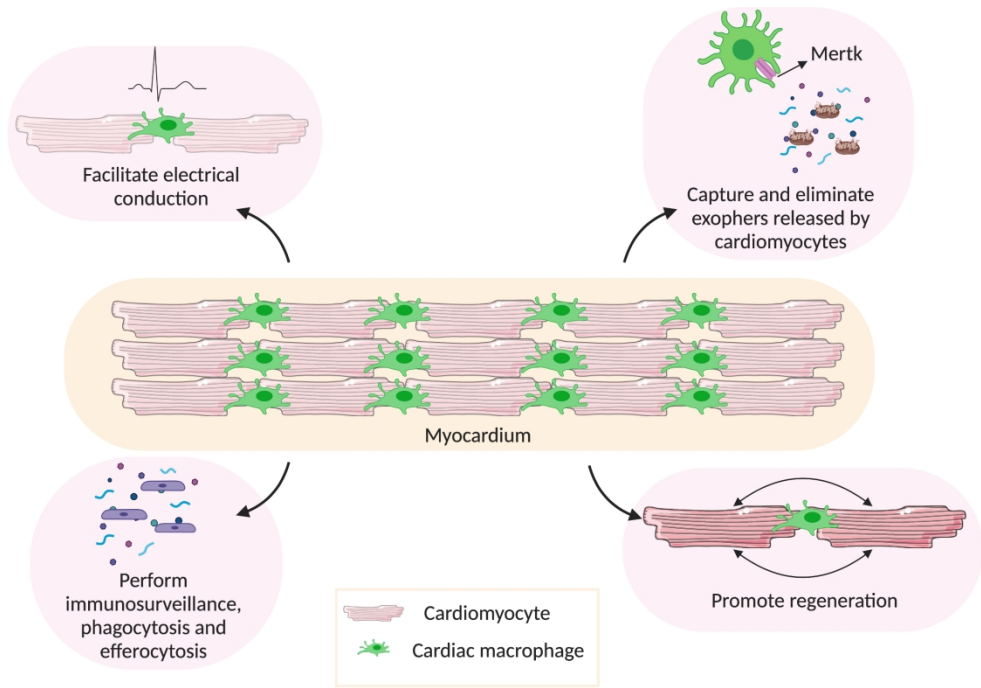


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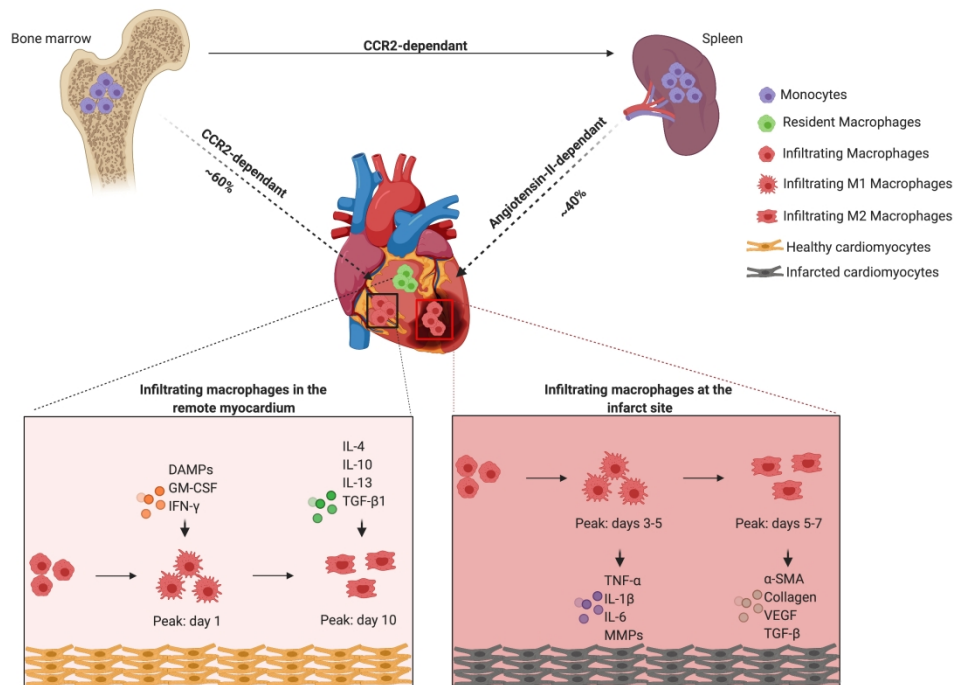


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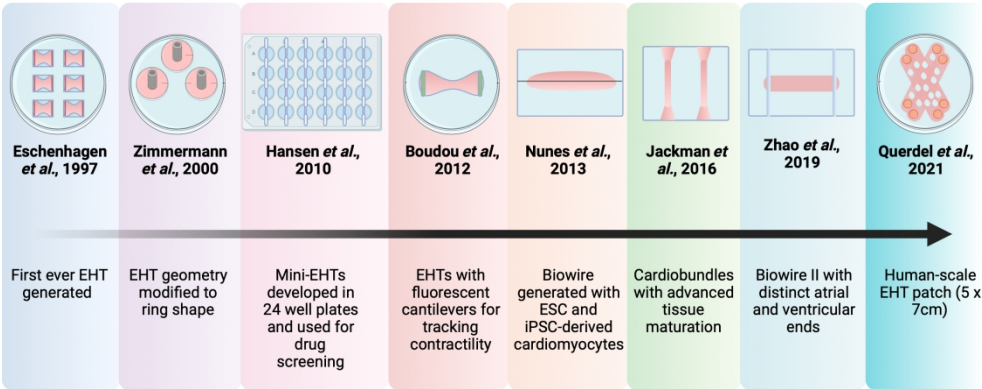


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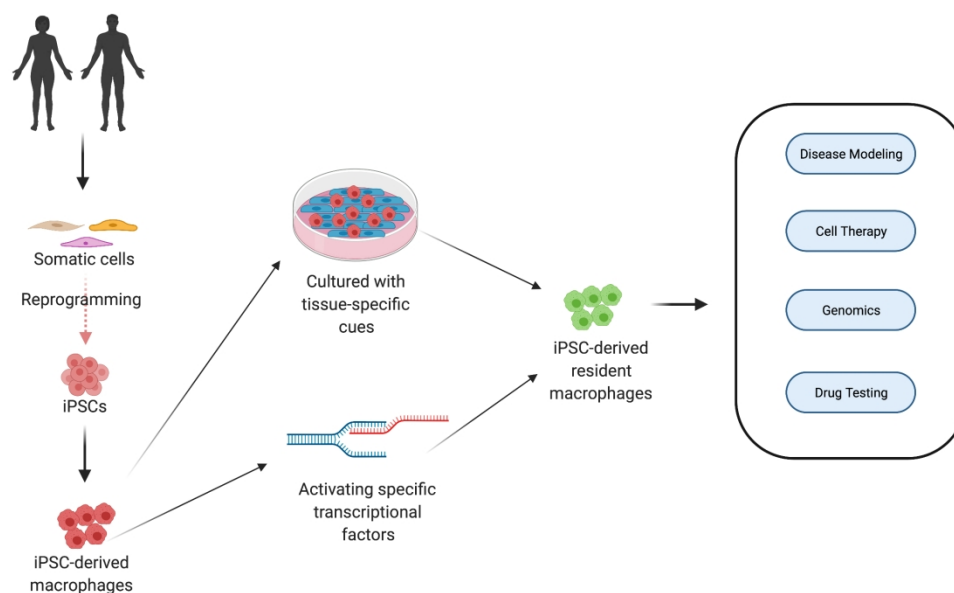


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Tissue Engineering

Author's Response to Decision Letter for (TEB-2021-0036)

Resident macrophages – a potential gamechanger in cardiac tissue engineering?

Dear Dr. Schenke-Layland,

Thank you for your recent communication regarding this manuscript, and the positive decision to consider it further given some revisions.

Many thanks to the reviewers for the time and attention to detail they have given to critique this manuscript. It is gratifying that they deem this work of interest to the readership of Tissue Engineering Part B: Reviews and recognize the effort we have made in organizing this manuscript. Their scrutiny pointed us towards reviewing the manuscript further and the suggested changes have been made. The revised version has greatly benefited from their critiques.

We are pleased to submit the revised manuscript with the titled modified as "Resident macrophages and their potential in cardiac tissue engineering". Thank you for allowing us the opportunity to address these comments of the reviewers. We have highlighted changes to the text in this revision in RED and specified their location.

All authors have read the manuscript and agreed to submit this revision to Tissue Engineering Part B: Reviews.

Thank you in advance for considering our manuscript.

Sincerely,

Michael Monaghan, Ph.D, B.Eng

Response to individual reviewer comments:

Reviewer 1

'This is a review article that describe about role of cardiac residential macrophage. There was no concrete idea how to utilize this macrophage for cardiac tissue engineering.'

As the reviewer rightly points out, the review article does describe critical functions of cardiac resident macrophages in healthy and infarcted myocardium. We did aim to give equal focus in putting forth potential ways of obtaining cardiac resident macrophages in vitro but agree that the initial submission fell short in fully discussing the utilization of macrophages for cardiac tissue engineering.

With this revised manuscript, we have added a significant discussion on cardiac resident macrophages in the maintenance of normal cardiac function and hence their potential to enhance tissue engineered cardiac models. While this focus may may not have been explicit in the original submission; we are confident that our revised manuscript now does this justice.

'In addition, there was no successful report for iPS derived macrophage.'

Derivation of macrophages from iPSCs is a fairly well-established protocol which we had discussed in the original submission. We emphasize this further in the revised manuscript. Specifically, references 84-93 and 95-97 show successful macrophage derivation from iPSCs.

'Title is misleading and should be changed to such as " The role of residential cardiac macrophage"'

We thank the reviewer for this suggestion. The title has been revised.

'The reviewer recommend to add the immuno-histological pictures of this macrophage in healthy heart and in diseased heart.'

We thank the reviewer for this suggestion. However, to our knowledge; there are no immunohistochemical images available of healthy human heart that depicts the presence of resident cardiac macrophages specifically, studies using healthy human heart tissue being fairly limited. There are, however, plenty of immunohistochemical images taken from murine heart where the depletion of resident macrophages in the myocardium can be seen during injury. Indeed, one of the themes of this review is that such biology is predominantly reported in such mouse models whereby the human biology can be entirely different which we discuss to model using iPSC derivatization of human resident macrophages to study this biology.

Reviewer 2 Mary Ann Liebert, Inc.,140 Huguenot Street, New Rochelle, NY 10801

'The review relies on the role of macrophage subpopulations in cardiac tissue physiology and in myocardial infarction, highlighting how interesting this knowledge is to be applied to cardiac tissue engineering. Although the article brings recent findings on macrophages and cardiomyocytes, very little has been explored on the engineering of cardiac tissue.'

We thank the reviewer for acknowledging the interest of using macrophages in cardiac tissue engineering and agree with the reviewer on how little this field has been explored. Their feedback is similar to that of reviewer one, which we have not made significant revisions to address.

'In addition, a review article with a similar proposal was recently published in this journal. Please, see: h (2020). Crosstalk between cardiac cells and macrophages: Insights from in vitro studies. Tissue Engineering Part B: Reviews.'

Many thanks to the reviewer for mentioning this paper by Hitscherich et. al which we do admire- it is very interesting and covers some topics that are similar to our manuscript. Regrettably, we had not come across it during the preparation of our manuscript due to its very recent publishing data. While some elements in our manuscript share a similar focus; we have shifted our theme to enable a clear distinction between the two. Our revised manuscript places strong focus on the different applications of macrophages in tissue engineering, with particular interest in cardiac tissue engineering. Additionally, we discuss the potential of bringing forward induced pluripotent stem cell (iPSC) and embryonic stem cell (ESC) derived macrophages because of their proven ability to differentiate into other tissue resident macrophages such as microglia and Kupffer cells. It is important to note, that the topic of resident macrophages is extremely topical at the moment- with this review, we aim to bring together existing knowledge in this field and how it can be put together to obtain cardiac resident macrophages.

'I suggest that the authors delve deeper into the tissue engineering models that have already been proposed and also which could be proposed in this area, since the tissue engineering with the immune system cell component is not so trivial.'

We thank the reviewer for this valuable suggestion and acknowledge the importance of tissue engineered models incorporating immune cells components. It shares similarities with the previous comments and we have added a significant amount of discussion to address this.

'Acronyms throughout the text that are not described'

Thanks to the reviewer for pointing this out, the revised manuscript addresses this.

'The title of last topic does not match the information'

Thanks to the reviewer for highlighting this, the revised manuscript includes modified subheading.

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